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Discovering Novel Serine Proteases Expressed in Prostate  
Cancer Using Phage Display

PRINCIPAL INVESTIGATOR: Jeonghoon Sun, Ph.D.

CONTRACTING ORGANIZATION: University of California, San Francisco  
San Francisco, California 94143-0962

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<b>13. ABSTRACT (Maximum 200 Words)</b> Quantitative RT-PCR and immunohistochemistry (IHC) experiments revealed that membrane-type serine protease 1 (MT-SP1), a member of the TTSP family, is specifically overexpressed in epithelial cancer cells and tissue samples. It was reported that in advanced stages of prostate, breast, and ovarian cancers MT-SP1 is not regulated by its endogenous inhibitor HAI-1. MT-SP1 plays diverse roles by activating pro-uPA, pro-HGF, and PAR-2. Taken together, MT-SP1 may be a key upstream factor involved in the ECM remodeling plasminogen activation cascade and in signal transduction cascades involved in cell transformation. Dysregulated MT-SP1 expression could promote the proteolytic activity of MT-SP1 resulting in a more invasive phenotype. We postulated that inhibition of its proteolytic activity might curtail the invasive phenotype. As MT-SP1 is a cell surface protein, we chose to target the protease with anti-MT-SP1 single chain monoclonal antibodies (scFvs) that we isolated using phage display. These scFvs potentially and selectively inhibit the protease activity of MT-SP1. Our initial in vivo studies revealed the sizes of xenografted human ovarian tumors in nude mice were significantly reduced following treatment with the inhibitory scFvs, indicating the therapeutic potential of these scFvs for the treatment of epithelial cancers in ovary, prostate and breast.				
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## Table of Contents

Cover.....	1
SF 298.....	2
Table of Contents.....	3
Introduction.....	4
Body.....	5
Key Research Accomplishments.....	8
Reportable Outcomes.....	9
Conclusions.....	9
References.....	9
Appendices.....	11

## Introduction

**Abbreviations:** scFv, single chain variable fragment; ECM, extracellular matrix, enzyme-linked immunosorbent assay; hMT-SP1-P, protease domain of human MT-SP1; MT-SP1, membrane-type serine protease 1; IHC, immunohistochemistry; TTSP, Type II transmembrane serine protease; RT-PCR, reverse transcriptase polymerase chain reaction.

**Membrane anchored serine proteases as new targets for treatment of cancer.** In the early phases of cancer, the growth of tumor cells remains confined to their original tissue boundaries. However, upon becoming malignant, cancer cells invade surrounding tissue by migrating from their tissue compartment and disseminating throughout the body. To penetrate through cellular barriers and metastasize, tumors employ invasion machinery. A hallmark of this machinery is dysregulated matrix-degrading proteases that digest extracellular matrix (ECM) proteins by cleaving peptide bond of specific amino acid sequences (Andreasen et al. 1997; Hanahan et al. 2000; Tanimoto et al. 1999; Wallrapp et al. 2000). An increasing amount of research has recently focused on a subgroup of the trypsin-like serine proteases that are directly anchored to plasma membrane on cell surface (Hooper et al. 2001; Szabo et al. 2003; Netzel-Arnett et al. 2003). These membrane-anchored proteases are anchored via a C-terminal transmembrane domain (Type I), via a glycosyl-phosphatidylinositol linkage, or via an N-terminal transmembrane domain (Type II or TTSP). Disruption or mutation of the genes encoding these proteases are associated with cancer. While these proteases show tightly regulated expression in normal cells, they are dysregulated and overexpressed during tumor progression. Membrane anchored serine proteases therefore represent a promising new and untapped source of potential targets for the development of new cancer therapeutics.

**MT-SP1.** To identify serine proteases expressed in the PC-3 cells, RT-PCR was performed with degenerate oligonucleotide primers that were designed using conserved protein sequences from the trypsin fold of serine proteases. Five independent serine protease cDNAs derived from PC-3 mRNA were sequenced, and a novel serine protease that we named membrane-type serine protease 1 (MT-SP1) was identified. The enzyme was independently identified by another group and named matriptase (Lin et al. 1997). MT-SP1 is a mosaic protein of 855 amino acids that contains a transmembrane signal anchor, two CUB domains, four LDLR repeats, and a serine protease domain. Immunoblotting and immunohistochemical analyses verified the presence of MT-SP1 in epithelial tissue of prostate tumor (Takeuchi et al. 1999; Sun et al. 2003) and breast tumor (Lin et al. 1999). As MT-SP1 is highly expressed in ovarian, breast, and prostate cancer cells and appears to be involved in tumor invasion, it is a potential target for cancer diagnosis and treatment (Takeuchi et al. 1999; Oberst et al. 2001). In addition to PC3 cells, MCF7 and OVCAR3 cells express significant amounts (10 – 50 fold compared to normal cells) of MT-SP1 mRNA (Robert Bok, personal communication). MT-SP1 has been shown to be overexpressed in human ovarian cancer specimens (Takeuchi et al. 1999; Tanimoto et al. 1999). The serine protease domain has been cloned into an *E. coli* expression vector and the recombinant version of the protein has been expressed, purified, and refolded to produce active enzyme (Takeuchi et al. 1999).

MT-SP1 may serve as a key upstream activator in the ECM remodeling plasminogen activation cascade. It has been shown to activate Latent urokinase plasminogen activator (uPA) and pro-human growth factor (HGF) *in vitro* (Lee et al. 2000). uPA participates in the plasmin-mediated remodeling of the extracellular matrix (ECM) surrounding tumors, the activation of latent HGF. HGF contribute in the stimulation of cancer cell growth and motility (Corps et al. 1997; Sowter et al. 1999). uPA contributes to invasion; the binding of uPA, itself a serine protease, to its receptor increases the proliferation of cancer cells *in vitro* (Fischer et al. 1998). Inhibition of uPA by an antisense oligonucleotide significantly reduces the intraperitoneal spread of ovarian cancer mouse xenograft models (Wilhelm et al. 1995). Another *in vitro* substrate of MT-SP1 was identified as protease activated receptor 2 (PAR-2). Recent

studies reveal that PAR-2 stimulation was associated with the transient phosphorylation of MAP kinase (Jikuhara et al. 2003). The highly similar PAR-1 and 3 were not activated by MT-SP1, suggesting that MT-SP1 could be a specific regulatory protease that affects signal transduction of cell proliferation (Takeuchi et al. 2000).

In normal cells, MT-SP1 activity is tightly regulated by the endogenous inhibitor hepatocyte growth factor activator inhibitor 1 (HAI-1) (Kirchhofer et al. 2003). HAI-1 is found predominantly in the epithelium of many tissue types and is up-regulated in injured or regenerative tissues. HAI-1 may suppress the growth and motility of cancer cells by inhibiting the generation of active uPA and active HGF by MT-SP1. The expression of MT-SP1, HAI-1, or the MT-SP1:HAI-1 ratio correlate with the clinical stage, histological grade, histological type, or clinical outcome of ovarian cancer patients (Oberst et al. 2002). Advanced-stage (III and IV) ovarian tumors were found to be more likely to express MT-SP1 in the absence of HAI-1. This indicates that an imbalance in the MT-SP1:HAI-1 ratio could be important in the development of advanced disease. Unregulated or dysregulated MT-SP1 expression could promote the proteolytic activity of MT-SP1, and, consequently, a more invasive phenotype. Taken together, these data suggest MT-SP1 may play a key regulatory role in the process of ovarian cancer progression.

**Antibodies as protease inhibitors.** Various protease inhibitors have been developed for numerous *in vitro* and *in vivo* applications. Although fairly effective as tools to study cancer progression and metastasis, small molecule inhibitors are plagued by problems with resistance and toxicity, while conventional macromolecular inhibitors can suffer from promiscuity and low activity (Coussens et al. 2002). The *E. coli* serine protease inhibitor ecotin has proven to be a valuable tool in the discovery of novel proteases. However its usefulness *in vivo* is limited, as it elicits an immune response when injected into mice. Moreover, since proteases are widely distributed in nature and many of them share nearly identical active site elements, we were concerned that ecotin would not be selective enough to distinguish among the closely related MT-SPs. The antibody scaffold is an attractive choice for the development of highly potent and selective inhibitors for this family since it can distinguish between closely related proteins. Furthermore, antibodies are regularly used diagnostic and therapeutic reagents. If successful, this method can be applied to other enzyme families such as matrix metalloprotease families and protein kinase families.

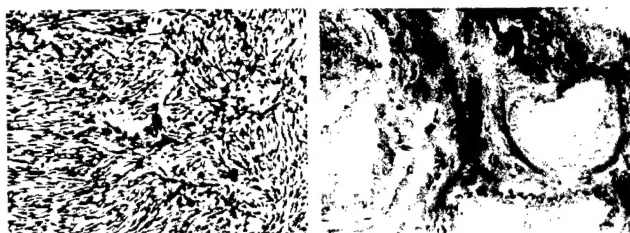
**The focus of FY2.** Two tasks were assigned for FY2. The first was to monitor inhibition of MT-SP1 to determine its role *in vivo* using the optimized scFvs. It was originally proposed that this would be performed with SCID mice transplanted with PC-3 cells, to show that MT-SP1 inhibition can be critical in intervention of epithelial cancers in prostate, breast, and ovarian cancers. Based on quantitative RT-PCR (Taqman™) analysis, it was evident that OVCAR-3 cell line produced higher levels of MT-SP1 mRNA. In the immunohistochemical staining analysis, MT-SP1 overexpression is more specific in ovarian cancer tissues than prostate cancer tissues (compare the Fig. 1 in this report with the Fig. 3 in the FY1 report). Thus, the pilot experiments were performed in collaboration with Dr. Robert Bok in Department of Medicine at UCSF with nude mice xenografted with ovarian tumors instead of prostate tumors as a step to clarify the role of MT-SP1. The second task was to find previously undiscovered serine proteases that might be involved prostate cancer using cDNA expression libraries derived from prostate tumor cell lines and RT-PCR. This project is underway and will be continued. In addition, the mode of inhibition of the most potent scFv, E2 has been kinetically characterized.

## **Body**

My mentor, Dr. Charles Craik, has provided outstanding training and a rich environment for learning protease biochemistry. Dr. Marc Shuman and Dr. Robert Bok in the department of Medicine in UCSF provided the facilities for studying the biology of prostate cancer and treating ovarian tumor xenografted

nude mice with scFvs. I have attended the monthly Proteases in Cancer Project meetings where I have had the opportunity to present data and interact with a diverse group of scientists including oncologists, hematologists, immunologists, structural biologists, and mechanistic enzymologists. Other helpful interactions included the weekly meetings held between Dr. Craik, Dr. Marc Shuman, postdoctoral researchers in Dr. Shuman's group, and myself. For my intellectual development, I have attended weekly seminar series offered by the departments of pharmaceutical chemistry, biochemistry and biophysics, and cellular and molecular pharmacology, and programs in chemistry and chemical biology, and quantitative biology and various annual lectureships at UCSF. I have attended the Experimental Biology 2003 Annual Meeting, ASBMB sector (April 11-15, San Diego, CA). This meeting allowed me to interact with other scientists who are working on similar areas of research. The US Army Medical Research and Material Command Prostate Cancer Research Program was acknowledged.

**Immunohistochemistry (IHC).** To determine the efficacy of using the scFvs as immunological reagents, IHC staining was performed on ovarian and prostate tumor tissues from randomly selected patients. These experiments revealed that only one of the six anti-MT-SP1 scFvs, S1, stained tissues to an appreciable extent. Noteworthy is the presence of antibody specific red chromogen in the glandular lumen in metastatic ovarian cancer tissue, suggesting that MT-SP1 is overexpressed and likely shed from the surface of the cancerous epithelial cell (Figure 1).

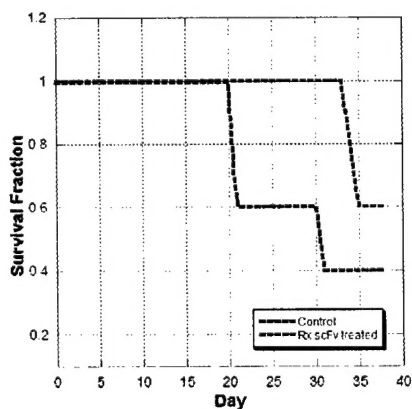


**Figure 1** Immunostaining of tissues with the S1 scFv detected MT-SP1 in a normal ovarian tissue (left) and in an ovarian cancer tissue (Right). Compare with the prostate tissues in FY1. results are consistent with our preliminary data.

#### **Quantitative RT-PCR (Taqman) analysis.**

**Immunohistochemistry (IHC).** The mRNAs from the Duke Ovarian Tissue Bank were obtained and relative mRNA levels were titrated using Taqman analysis. Most of the patients (22/24) were stage IIIC-IV. Tissues from five normal ovaries were used as controls. The relative message level for MT-SP1 in ovarian cancer was  $3.287 \pm 0.397$  ( $n = 24$ ) while that value from normal ovary was  $0.000 \pm 0.000$  ( $n = 5$ ). These

**Suppression of xenografted ovarian cancer by the anti-MT-SP1 scFvs (Fig 2 and Fig 3).** An *in vivo* pilot study has been performed to assess the efficacy of the anti-MT-SP1 scFvs in xenografted tumors of nude mice. This study was considered as a pilot study to clarify the role of MT-SP1 before PC-3 cell



**Figure 2.** Simple survival plot of mice treated by therapeutic scFv cocktail and control scFv.

transfected mouse experiments were performed. Human OVCAR-3 cells were injected into 10 nude mice subcutaneously. After 14 days, when tumors were well established, the mice were separated into a therapy group (5 mice) and control group (5 mice). An inhibitor cocktail, a combination of three scFvs (0.4  $\mu$ g of E2, 1.2  $\mu$ g of S1 and 0.4  $\mu$ g of S4) was injected into each mouse in the therapy group 3 times a weeks subcutaneously. A control scFv SA6 (2  $\mu$ g) that neither binds nor inhibits MT-SP1 was injected into each mouse in the control group. Before the animal trial all of scFvs were purified using FPLC and confirmed free of endotoxin contamination. Tumors were measured weekly using calipers and were documented photographically (Fig. 3). No overt toxicity was observed in any of mice. After reaching euthanasia criteria (or at spontaneous expiration), animals were sacrificed and tumors dissected and weighed. Peritoneal surfaces and organs were examined for macro-



and microscopic metastases. The mice treated with inhibitory scFvs survived for a mean of 38 days while the mice treated with the control scFv survived for a mean of 30 days (Fig. 2). The mice in the control group developed larger subcutaneous tumors and more ascites (Fig. 3). These results indicate a therapeutic effect from these scFvs and strongly suggest the experiment be repeated with larger number of animals. Since the weekly doses administered were minimal, we could increase the dose and injection frequency of the scFvs. Because each scFv revealed a different inhibition mechanism, binding affinity and binding mode, the efficacy of each scFv should be individually tested.



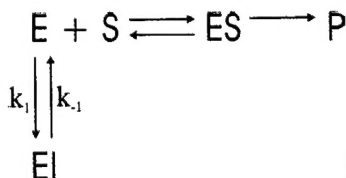
**Figure 3.** Xenografted human ovarian cancer progression of the nude mice. Five mice (Rx 1 ~ Rx 5) were treated with therapeutic scFv cocktail (E2 + S1 + S4). E2 scFv is the most potent inhibitor. S4 is a rapidly binding inhibitor. S1 is believed to be an allosteric inhibitor. All of these scFvs are very selective MT-SP1 inhibitors. Another five mice (control 1 ~ control 5) were treated with a control scFv that does not inhibit MT-SP1 at all. Compared to mice treated with therapeutic scFvs, the mice treated with the control scFv have developed significantly larger tumors. Starting week three ascites developed in the control group (control 1 and 4 circled in red). Control mice 1 and 4 died after week three.

**Creating cDNA library derived from PC-3 and OVCAR-3 cells.** Two cDNA library are in the process of being created through reverse transcription of poly-A-enriched mRNAs from human PC-3 cells and OVCAR-3 cells. The cDNA products were tagged with random primed elongation and PCR amplification using a standard protocol. The resulting pool of inserts was selected based on their size. Small-size DNA inserts were preferentially amplified during a PCR reaction and the efficacy of each step is being monitored by PCR. Once the biased amplification problems are solved library construction can be continued. We have been focused on characterizing the scFvs rather than exploring new targets. We would like to have an understanding of how these inhibitors function before we focus our attention on other TTSPs.

**Kinetic characterization of the scFv E2 (Fig 4).** E2 and a MT-SP1 substrate spectrozyme tPA were incubated 1h and protease domain of human MT-SP1 (hMT-SP1-P) was added to obtain the progressive curves of hMT-SP1-P activity. This experiment was performed at 10 different E2 concentrations and 4 different spectrozyme tPA concentrations. Each curve was fit in the integrated first order rate equation that describes the slow establishment of equilibrium between enzyme and inhibitor. The equation is

$$P = V_{st} + (V_o - V_s)(1 - e^{-k_{obs}t}) / k_{obs}$$

where P is product ratio,  $V_0$  is the initial steady-state velocity,  $V_s$  is the final steady-state velocity,  $t$  is time, and  $k_{obs}$  is the apparent first order rate constant. Thus, 40 different  $k_{obs}$  of hMT-SP1-P inhibition by E2 were obtained from 40 combinations of E2 and Spectrozyme tPA concentrations. It was postulated that the mechanism would be one-step competitive inhibition as represented in the scheme 1.

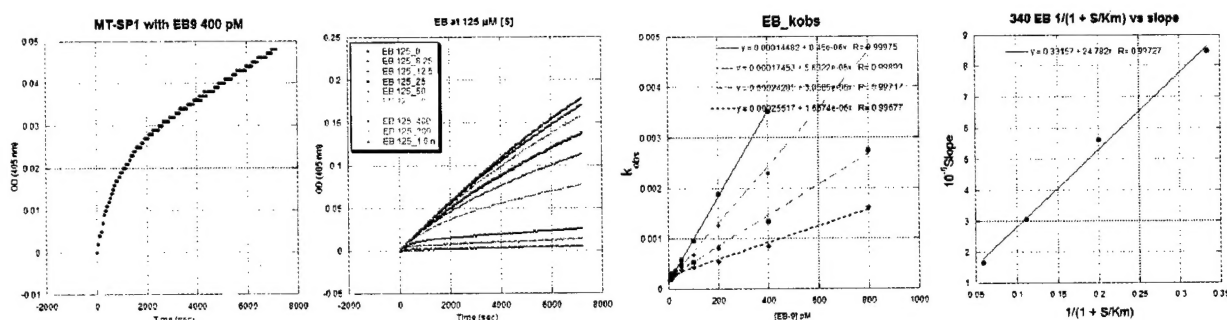


Scheme 1

The equation linking  $k_{obs}$  with  $k_{-1}$  and  $k_1$  is (Fig. 4 Bottom left):

$$k_{obs} = k_{-1} + k_1[I]/(1 + [S]/K_m)$$

where  $k_1$  is association rate and  $k_{-1}$  is dissociation rate. At fixed  $[S]$ , there exist linear relationship between  $k_{obs}$  and  $[I]$ . From this relationship  $k_{-1} = 2.04 \times 10^{-4} s^{-1}$  was obtained. By fitting slope of this graph to  $1/(1 + S/K_m)$ ,  $K_m = 34 \mu M$ . The association rate  $k_1 = 2.48 \times 10^8 M^{-1}s^{-1}$  (Fig. 4 far right). This kinetic characterization reveals that E2 is extremely tight-binding, competitive, rapidly associating and slowing dissociating MT-SP1 inhibitor.



**Figure 4.** Kinetic characterization of the scFv E2. (Far left) Progressive curves of MT-SP1 activity at 250  $\mu M$  of spectrozyme tPA, 400 pM of E2 (= EB9) and 10 pM of MT-SP1. (Left) Progressive curves of MT-SP1 activity at 125  $\mu M$  of spectrozyme tPA with 10 different E2 concentration ranging from 0 to 1600 pM. Similar curves were obtained at 62.5, 250, and 500  $\mu M$  of spectrozyme tPA concentrations. (Right).  $k_{obs}$  vs  $[E-2]$  at four different [spectrozyme tPA]. Red line: 62.5  $\mu M$ , blue line: 125  $\mu M$ , green line: 250  $\mu M$ , and black line :500  $\mu M$ . (Far right) slope of ( $k_{obs}$  vs  $[E-2]$ ) vs  $k_1/(1+[S]/K_m)$ ,  $K_m = 34 \mu M$ .

### Key research accomplishments

- Complete kinetic characterization of E2 suggesting that E2 is an extremely tight-binding, competitive, rapidly associating and slowing dissociating MT-SP1 inhibitor.
- Isolation of anti-human MT-SP1 scFvs that inhibit the protease activity of MT-SP1 potently and specifically.
- The scFv S1 was sensitive to detect specific overexpression of MT-SP1 in normal ovary and ovarian cancer tissues.
- Treatment of ovarian tumor xenografted mice with scFv combination revealed a proof of principle that



inhibiting MT-SP1 could be an efficacious modality to treat epithelial cancer progression and metastasis. A similar result is predicted in prostate tumors where MT-SP1 is overexpressed.

### Reportable outcomes

- Previously I reported that the results of FY 1 were in the process of publication in *Biochemistry*. It was published in FY2 (Jeonghoon Sun, Jaume Pons, and Charles S. Craik, "Potent and Selective Inhibition of Membrane-Type Serine Protease 1 by Human Single-Chain Antibodies", *Biochemistry*, 2003, 892 - 900). I attached the reprint. The US Army Medical Research and Material Command Prostate Cancer Research Program was acknowledged.
- In FY2, I was invited as a speaker in Experimental Biology 2003 Annual Meeting, ASBMB sector (April 11-15, San Diego, CA) and presented the results of FY1 and part of FY2. DOD PCRP was acknowledged. At the end of this report the abstract was attached.

### Conclusions

Our preliminary data and reported results of others indicate that MT-SP1 may be a critical element in the progression of epithelial tumors of the prostate, breast, and ovary. To gain insight into the roles that MT-SP1 plays in epithelial cancers, a general method for selectively and potentially blocking its proteolytic activity was established. Since MT-SP1 is a member of a very closely related family of TTSPs, it is important to selectively inhibit only MT-SP1 activity, while not affecting other TTSPs that frequently coexpress and share a common substrate binding site. The preference for Arg/Lys in the S1 pocket of the substrate binding site exhibited by this family of over 20 enzymes limits the success of small molecule approaches to selective inhibition of MT-SP1 in a biological setting. However, the potential difficulties were overcome by developing human scFvs as very specific MT-SP1 inhibitors. Using phage display human scFvs were isolated. These scFvs inhibit proteolytic activity of MT-SP1 potently and extremely selectively. It was demonstrated that inhibition of MT-SP1 by these scFvs resulted in suppressing ovarian tumors in mice where human ovarian tumors were xenografted. MT-SP1 overexpression is common to ovarian and prostate tumors. We chose this xenogenic ovarian cancer model as a pilot study because of the low background level of MT-SP1 in the normal ovary as compared with the normal prostate. The pilot study was performed in relatively small number of animals with minimal doses of the inhibitory scFvs. Thus, preclinical trials in this model and human prostate tumor model in SCID mice should be performed with statistically significant number of animals, increased doses, optimized reagents, improved injection methods, and various tumor models including prostate cancer models. These scFv-based regimens could conveniently be developed as diagnostics and/or prognostics such as IHC and ELISA reagents to follow up individual cases of ovarian cancer patients. Also these scFv reagents can be developed as immunoblotting or immunoprecipitation reagents that could be a valuable tool to explore the role of MT-SP1. Taken together, this approach will address the program's goals in the areas of preclinical development of targeted therapeutics and tumor biology by establishing a general method that permits a thorough analysis of the contribution that MT-SP1 makes to a complex biological process of epithelial cancer. Furthermore, the proof of principle that antibodies can be potent and selective inhibitor of protease activity can be applied to other members of TTSPs, the number of which is still increasing and are found in various tumors in prostate, breast, colon, and ovary.

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## Appendices

- Jeonghoon Sun, Jaume Pons, and Charles S. Craik, "Potent and Selective Inhibition of Membrane-Type Serine Protease 1 by Human Single-Chain Antibodies", *Biochemistry*, **2003**, 892 -900. (This paper was submitted in FY1. But that time it was "in press" status).
- Conference abstract in in Experimental Biology 2003 Annual Meeting, ASBMB sector (April 11-15, San Diego, CA): A novel class of protease inhibitor: potent and selective inhibition of membrane-type serine protease 1 by human single-chain antibodies, Jeonghoon Sun and Charles S. Craik

Department of Pharmaceutical Chemistry, University of California, San Francisco, 513 Parnassus, San Francisco, CA 94143, Developing selective and potent protease inhibitors is challenging due to frequent coexpression of other similar proteases. The antibody scaffold is an attractive choice for the development of highly potent and selective inhibitors. A phage-displayed antibody library was screened against a cancer-associated protease, membrane-type serine protease 1 (MT-SP1). Six inhibitory antibodies were selected and had  $K_i$ 's against human MT-SP1 ranging from 50 pM to 2 nM. Two antibodies had 800-fold and 1500-fold selectivity for human MT-SP1 when tested against mouse MT-SP1 that exhibits 87% sequence identity. These antibodies also did not inhibit other closely related serine proteases including factor Xa, thrombin, kallikrein, tPA, and uPA. Binding kinetics of the antibodies revealed association rates as high as  $1.2 \times 10^7 \text{ s}^{-1}\text{M}^{-1}$  and dissociation rates as low as  $3.8 \times 10^{-4} \text{ s}^{-1}$ . One antibody detected denatured MT-SP1 with no cross reactivity to other proteases in PC3 cells. Another antibody recognized the enzyme in human prostate tissue samples for immunohistochemical staining. The mode of binding between the antibodies and MT-SP1 was analyzed by competition ELISA using three different inhibitors. These antibodies constitute a new class of highly selective protease inhibitors for exploring the biological roles of proteases.

## Potent and Selective Inhibition of Membrane-Type Serine Protease 1 by Human Single-Chain Antibodies<sup>†</sup>

Jeonghoon Sun, Jaume Pons,<sup>‡</sup> and Charles S. Craik\*

Department of Pharmaceutical Chemistry, University of California, San Francisco, 513 Parnassus, San Francisco, California 94143

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**ABSTRACT:** Specific human antibodies targeting proteases expressed on cancer cells can be valuable reagents for diagnosis, prognosis, and therapy of cancer. To this end, a phage-displayed antibody library was screened against a cancer-associated serine protease, MT-SP1. A protein inhibitor of serine proteases that binds to a defined surface of MT-SP1 was used in an affinity-based washing procedure. Six antibodies were selected on the basis of their ELISA profiles and ability to serve as useful immunological reagents. The apparent  $K_i$ , indicative of the potency of the antibodies at inhibiting human MT-SP1 activity, ranged from 50 pM to 129 nM. Two of the antibodies had approximately 800-fold and 1500-fold selectivity when tested against the most homologous serine protease family member, mouse MT-SP1, that exhibits 86.6% sequence identity. Surface plasmon resonance was used as an independent means of determining the binding constants of the six antibodies. Association rates were as high as  $1.15 \times 10^7 \text{ s}^{-1} \text{ M}^{-1}$ , and dissociation rates were as low as  $3.8 \times 10^{-4} \text{ s}^{-1}$ . One antibody was shown to detect denatured MT-SP1 with no cross reactivity to other family members in HeLa or PC3 cells. Another antibody recognized the enzyme in human prostate tissue samples for immunohistochemistry analysis. The mode of binding among the six antibodies and the protease was analyzed by competition ELISA using three distinctly different inhibitors that mapped the enzyme surface. These antibodies constitute a new class of highly selective protease inhibitors that can be used to dissect the biological roles of proteolytic enzymes as well as to develop diagnostic and therapeutic reagents.

Proteases are involved in all stages of cancer progression including growth, angiogenesis, invasion, migration, and metastasis (1–6). At the crux of cancer pathogenesis is metastasis during which unregulated proteases lead to invasive remodeling of the extracellular matrix (7–10). Therefore, inhibitors targeting these proteases have been developed. Although partially effective as tools to study cancer progression and metastasis, small-molecule inhibitors are plagued by problems with resistance and toxicity, while macromolecular inhibitors can suffer from promiscuity and low activity. Moreover, proteases are widely distributed in nature comprising approximately 2% of the entire human genome (11). This omnipresence of proteases with nearly identical active site elements creates the need to develop a general method for producing potent, highly selective inhibitors.

Validating a protease as an appropriate therapeutic target for cancer is a challenging task. The recent discovery of the

type II transmembrane serine proteases that have been detected at high levels in various cancer cell lines has underscored their potential role in cancer (4, 5, 8). One member of this family, membrane-type serine protease 1 (MT-SP1),<sup>1</sup> has been implicated as a key protease in triggering the plasminogen proteolytic cascade, a central pathway in cancer progression and metastasis. MT-SP1 is expressed in human tissues such as prostate, breast, and ovary (12–15). MT-SP1 is a mosaic protein of 855 amino acids and is composed of a short cytosolic N-terminal region, a transmembrane signal anchor, several protein–protein interaction domains, and a C-terminal protease domain. The serine protease domain at the C-terminus (hMT-SP1-P) is strategically positioned on the cell surface to allow for efficient interaction with other proteins, thus making it a potentially recognizable epitope and a direct target for regulation of the protease activity (12).

Since MT-SP1 is expressed on the cell surface, where large molecules can easily access the protease domain, human monoclonal antibodies (MAbs) were explored for their application in inhibitor design. As therapeutic reagents, human MAbs can be far less toxic and more selective inhibitors than both small molecule and other macromolecular inhibitors. Furthermore, MAbs can easily be developed

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\* To whom correspondence should be addressed. Mailing address: 513 Parnassus Box 0446, University of California, San Francisco, San Francisco, CA 94143-0446. Telephone number: 415-476-8146. Fax: 415-502-8298. E-mail address: craik@cgl.ucsf.edu.

<sup>‡</sup> Current address: Rinat Neuroscience corporation, 3155 Porter Drive, Palo Alto, CA 94304.

<sup>1</sup> Abbreviations: <sup>1</sup>MT-SP1, membrane-type serine protease 1; scFv, single chain variable fragment; ELISA, enzyme-linked immunosorbent assay; hMT-SP1-P, protease domain of human MT-SP1; YT, yeast extract; tryptone.

as general tools to further investigate unidentified roles of MT-SP1 (16). MAbs are heterodimeric proteins, and the light- and heavy-chain variable domains can be linked together by a peptide spacer and expressed as one chain. This chimeric protein is referred to as a single chain Fv (scFv) (17, 18). In comparison to full-length antibodies, the scFv is a small monomeric single chain that is more amenable to manipulation by genetic recombination, large-scale bacterial protein expression, and phage display. Yet scFvs retain antibody-like specificity and affinity, and the scFv scaffold can be expanded to the Fab or the full IgG as needed.

Avoiding the restraints of conventional hybridoma approaches, phage display is an efficient method that can be inventively applied for direct selection of human scFvs (19, 20). As the quality of the primary library is certainly one of the most important components, we chose a phage-displayed human combinatorial scFv library (21). This library was synthetically constructed mimicking the human antibody repertoire in terms of structure, amino acid sequence diversity, and germline usage with seven  $V_H$  and seven  $V_L$  families covering greater than 95% of the human antibody diversity (22–24). Containing approximately  $2 \times 10^9$  members, this library provides large diversity with low redundancy (21).

We report here *in vitro* selection of human scFv inhibitors of the catalytic domain of MT-SP1 via phage display aided by an affinity washing procedure. Enzyme inhibition assays, kinetic analysis of binding, immunoblotting, and immunohistochemical staining were performed for characterization of these scFvs. Well-characterized small molecule and macromolecular serine protease inhibitors were used in a competition ELISA to probe the binding interactions of the scFvs to the protease. Highly potent, inhibitory scFvs of various binding modes and selectivity were developed for human MT-SP1 to assist in evaluating its physiological role.

## EXPERIMENTAL PROCEDURES

**Media and Buffers.** The components of reaction media and buffers used are listed below. *BBS*: 200 mM boric acid, 150 mM NaCl, 2 mM EDTA, 20% sucrose, pH 8.0. *HBS-EP*: 10 mM HEPES, 150 mM NaCl, 3 mM EDTA, 0.05% polysorbate 20 (Tween 20), pH 7.4. *LB*: 1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0. *LB-1*: LB, 34  $\mu\text{g mL}^{-1}$  chloramphenicol, 1% glucose. *NP-40 lysis buffer*: 50 mM Tris-HCl, 150 mM NaCl, 1.0% NP-40. *PBS*: 0.9% NaCl, 10 mM sodium phosphate, pH 7.2. *MPBS*: PBS, 5% nonfat milk, pH 8.0. *PBST*: PBS, 0.05% Tween 20. *PPB*: 30 mM Tris-HCl, 1 mM EDTA, 20% sucrose, pH 8.0. *TBS*: 150 mM NaCl, 10 mM Tris-HCl, pH 7.5. *TBST*: TBS, 0.05% Tween 20; *MTBST*: TBST, 5% nonfat milk. *TBSTCa*: TBST, 1 mM  $\text{CaCl}_2$ . *YT*: 0.5% tryptone, 0.5% yeast extract, 0.5% NaCl. *YT-1*:  $2 \times$  YT, 34  $\mu\text{g mL}^{-1}$  chloramphenicol, 0.1 mM isopropyl D-thiogalactoside (IPTG). *YT-2*:  $2 \times$  YT, 34  $\mu\text{g mL}^{-1}$  chloramphenicol, 1% glucose. *YT-3*:  $2 \times$  YT, 34  $\mu\text{g mL}^{-1}$  chloramphenicol, 0.1% glucose, 1 mM IPTG; *YT-4*:  $2 \times$  YT, 34  $\mu\text{g mL}^{-1}$  chloramphenicol.

**Affinity Selection.** All experiments were performed at room temperature unless specifically mentioned. For round 1,  $2 \times 3$  wells on a Maxisorp plate (Nalgenunc, Rochester, NY) and one well for control in another Maxisorp plate were coated with 10  $\mu\text{g}$  of recombinant hMT-SP1-P (12) per well

in 300  $\mu\text{L}$  of 50 mM  $\text{NaHCO}_3$  buffer (pH 9.6), incubated 16 h at 4  $^\circ\text{C}$ , and washed with PBS. In the control well, 100  $\mu\text{L}$  of Spectrozyme tPA (100  $\mu\text{M}$  in 50 mM NaCl, 50 mM Tris-HCl (pH 8.8), and 0.01% Tween 20) was added to test the enzyme activity. The other antigen-coated wells were treated with a blocking buffer, MPBS, for 2 h with gentle shaking. The blocking solution was removed and rinsed twice with PBS. Meanwhile, 450  $\mu\text{L}$  of the scFv phage library (21) was mixed with 450  $\mu\text{L}$  MPBS containing 0.1% Tween 20. The preblocked phage mix was transferred into each well and incubated for 1 h. After the phage solution was removed, three of the wells for standard wash were rinsed five times with PBST and five times with PBS. The other three wells for the ecotin wash were rinsed with 100 nM ecotin-containing PBST five times and PBS five times. The ecotin variant used for this affinity wash was an engineered dimer that contains the sequence TSSRRA at positions 81–86 and an arginine at position 70. This mutant ecotin inhibits MT-SP1 at 50 pM using 100  $\mu\text{M}$  of the substrate Spectrozyme tPA (American Diagnostica, Greenwich, Connecticut). *E. coli* TG1 cells were added, and the mixture was incubated for 45 min at 37  $^\circ\text{C}$  with gentle shaking. VCSM13 helper phage was added to each well and the mixture was incubated for 2 h at 37  $^\circ\text{C}$ . Aliquots from each well were set aside for titration and the rest was incubated for 16 h.

For round 2, 2 wells on a Maxisorp plate were coated with hMT-SP1-P and rinsed as in round 1 and blocked with 5% MPBS for 2 h. The TG1 cells were spun for 10 min at 2200g at 4  $^\circ\text{C}$ . Supernatants from the three wells of the standard wash were combined, as were the supernatants from the three wells of the ecotin wash. Each 165  $\mu\text{L}$  of supernatant from the ecotin pool and the standard pool was added separately into each 165  $\mu\text{L}$  of MPBS with 0.1% Tween 20. The two mixtures were incubated for 2 h with gentle shaking. Aliquots from each well were set aside for round 2 input titer. The blocking solution from the Maxisorp plate was removed, and each pre-blocked phage mix (300  $\mu\text{L}$ ) was transferred into each coated well and incubated for 1 h. After the phage solution was removed, one well (standard pool) was rinsed five times with PBST, incubated with PBST ( $5 \times 5$  min), and rinsed five times with PBS. The other well (ecotin pool) was rinsed five times with PBST, incubated with 100 nM ecotin in PBST ( $3 \times 5$  min), and rinsed five times with PBS. VCSM13 helper phage were added to each well, and the infected cells were transferred into shake flasks with 50 mL YT-1 and incubated for 16 h at 37  $^\circ\text{C}$  with gentle shaking. The overnight culture (1 mL) from the round 2 panning was stored in 15% glycerol at  $-80$   $^\circ\text{C}$  as a polyclonal selection pool. The remaining 49 mL was spun, the cells were harvested, and the plasmid DNA was purified. A 864 bp *Xba I/EcoR I* fragment was purified and ligated into pMx7FH vector (25). The ligation mixture was electroporated into JM83 cells, and the cells were spread onto an LB-1 agarose plate that was incubated 16 h at 37  $^\circ\text{C}$ . Colonies were chosen, grown, and preserved in 15% glycerol at  $-80$   $^\circ\text{C}$ .

**Microexpression of ScFv.** YT-2 was inoculated with scFv glycerol stock and incubated at 37  $^\circ\text{C}$  for 3 h with shaking at 250 rpm. Expression was induced at an  $A_{600}$  of 0.5 by adding 100  $\mu\text{L}$  YT-3, and the mixture was incubated for 4 h at 30  $^\circ\text{C}$  with gentle shaking. Cells were spun down, resuspended in 125  $\mu\text{L}$  of ice-cold BBS, incubated at 4  $^\circ\text{C}$



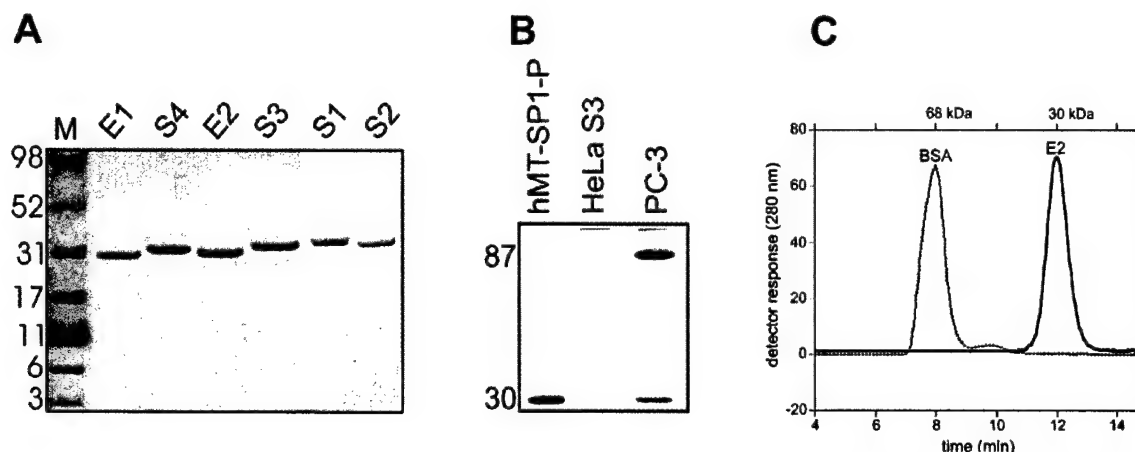


FIGURE 1: SDS-PAGE and western analyses. (A) SDS-PAGE analysis of the six scFvs after IMHC. This experiment was performed in reducing conditions. The molecular weight markers are shown in kilodaltons in the lane M. Other lanes show the scFvs that were used for further analyses. These scFvs produce a pure single band around 30 kDa. (B) Immunoblot analysis. S2 was used as the primary antibody. The recombinant hMT-SP1-P is shown at 30 kDa. HeLa S3 cells did not express MT-SP1. PC-3 cells produced full-length MT-SP1 at 87 kDa, along with the cleaved protease domain at 30 kDa. (C) Elution profiles of BSA (68 kDa) and E2 (30 kDa). BSA and E2 were run separately but represented in the same graph. Other scFvs, including E1, S1, S3, and S4, also eluted after approximately 12 min and only one major peak was observed as for E2.

for 16 h with shaking, and spun down. The supernatant was then transferred to a new 96 well plate.

**ELISA.** A Maxisorp plate was coated with 0.5  $\mu$ g of hMT-SP1-P per well in 100  $\mu$ L of 50 mM NaHCO<sub>3</sub> buffer (pH 9.6) and incubated overnight at 4 °C. The antigen in solution was removed, and the plate was rinsed with TBST and blocked for 1 h with MPBS. The scFv microexpressed supernatants (50  $\mu$ L) (or various concentrations of isolated scFvs (50  $\mu$ L)) and MPBS (50  $\mu$ L) were added to each well and incubated for 1.5 h. Wells were then washed 5 times with TBST. Anti-Flag M2-HRP conjugate (100  $\mu$ L, 1:15000 in TBST, Sigma, St. Louis, MO) was added to each well and incubated for 1 h. After 5 washes with TBST, peroxidase substrate BM blue POD substrate (Roche Diagnostics, Indianapolis, IN) was added, and the reactions were developed for 10 min and then stopped with the addition of 1 M H<sub>2</sub>SO<sub>4</sub> (100  $\mu$ L). The absorbance was determined at 450 nm using a microplate reader.

**ScFv Expression and Protein Purification.** A single bacterial colony containing the pMx7FH plasmids of each scFv was inoculated into YT-4 (10 mL), and the cells were grown overnight. One liter of YT-4 was inoculated with the scFv culture (2 mL) and incubated at 30 °C, 250 rpm. Expression was induced at an  $A_{600}$  of 0.5 by addition of IPTG to a final concentration of 0.5 mM. Incubation was continued for 16 h with vigorous shaking. Cells were harvested, resuspended in precooled PPB, and incubated for 30 min at 4 °C. Cells were collected and resuspended in precooled BBS and incubated overnight at 4 °C. The PPB and BBS supernatants were combined and spun to pellet more bacterial debris. The supernatant was filtered through a 0.2  $\mu$ m filter. PCR was performed on the bacterial pellet for DNA sequencing. The His-tagged scFv fragments were purified by immobilized-metal ion affinity chromatography (IMAC) using Ni-NTA Agarose (Qiagen, Valencia, CA) according to the manufacturer's protocol and analyzed by SDS-PAGE and size exclusion chromatography (Figure 1c) using Superdex75 FPLC column (Amersham Pharmacia Biotech, Peapack, NJ). Each scFv was eluted in the MT-SP1 enzyme

assay buffer containing 50 mM NaCl, 50 mM Tris-HCl (pH 8.8), and 0.01% Tween 20.

**Immunoblot Analysis.** Samples including pure recombinant hMT-SP1-P and lysates of the HeLa S3 and PC-3 (26) cells were analyzed using a standard immunoblot protocol as previously reported (13) using the screened scFvs as primary antibodies and the anti-flag M2-HRP conjugate as a secondary antibody. The samples were boiled for 2–5 min at 100 °C in the presence of SDS and 2-mercaptoethanol. Electrophoresis was performed under a reducing conditions using SDS and 2-mercaptoethanol. The substrate for HRP was ECL (Amersham Pharmacia Biotech, Peapack, NJ).

**Inhibition Assay.** The initial reaction velocities of human MT-SP1, mouse MT-SP1, and trypsin were measured in 50 mM NaCl, 50 mM Tris-HCl (pH 8.8), and 0.01% Tween 20 with 100  $\mu$ M Spectrozyme tPA (methyl-sulfonyl-D-cyclohexyltyrosyl-glycyl-L-arginine-*p*-nitroanilide) as a substrate and various concentrations of scFvs. Inhibition constants were evaluated by fitting to the equation for tight-binding inhibitors (27).

**Binding Kinetics.** The association and dissociation rate constants were obtained by surface plasmon resonance using a BIAcore Biosensor 2000 (Uppsala, Sweden). The hMT-SP1-P (ligand), in 10 mM glycine-HCl, pH 3.0, was covalently immobilized onto a CM5 chip according to the manufacturer's protocol. The hMT-SP1-P was stable for 7 days at pH 3.0, and its proteolytic activity was recovered in HBS-EP running buffer. Low-density surfaces (<100 RU) and high flow rates (50  $\mu$ L min<sup>-1</sup>) were used for kinetic parameter determinations. The scFvs (analytes) were dialyzed in running buffer HBS-EP to minimize bulk refractive index differences. Surface regenerations were performed with a 25  $\mu$ L pulse of ImmunoPure Gentle Ag/Ab elution Buffer (Pierce, Rockford, IL). The reference channel was treated using the same chemistry as the ligand coupled surface. The sensorgram of the reference surface was subtracted from the ligand conjugated surface for each injection, and multiple injections of running buffer were averaged and subtracted from the whole data set to remove mechanical noise. A series



Table 1: Amino Acid Sequences of the CDR3<sup>a</sup>

ScFv	V <sub>L</sub> template	V <sub>L</sub> sequence	V <sub>H</sub> template	V <sub>H</sub> sequence
E1	Vκ1	QYSNFPSTF	VH3	LPYYGRPGGYRFFDV
E2	Vκ1	QHGNLPYTF	VH3	PYLTPQRRGPQNVSPFDN
S1	Vλ1	SYDGNSQNF	VH3	RGYGVWSGWHGYHFDY
S2	Vκ2	QMSNFPMTF	VH3	HKTNSFRHLRATFPDFDV
S3	Vλ2	LSYDRFLT	VH1A	QHRGHTYGRGYKIFFDP
S4	Vλ1	SRDISQY	VH1A	FHIRRYRSGYYDKMDH

<sup>a</sup> The CDR3 region of V<sub>L</sub> were randomized with 4–21 residue variations and the CDR3 of V<sub>H</sub> with 7–30 residues. All combinations of other variable regions were fixed in 49 templates from the master repertoires comprising 7 light chains and 7 heavy chains (21).

of analyte concentrations ranging from 100 to 0.1 nM were injected, and data were fit globally to obtain association and dissociation kinetic parameters using a 1:1 Langmuir binding model in the BIAevaluation 3.1 software. All kinetic rates reported are the average of two independent determinations on three different surfaces. The data for the low affinity binder S2 were estimated by steady-state affinity fitting using the same software. No mass transfer problems were detected by injecting analytes at 5, 15, and 75  $\mu\text{L min}^{-1}$  on low-density surfaces. Different injection times (0.5, 5, 20 min) of 100 nM analyte did not show any evidence of linked reactions.

**Competition ELISA.** A competition ELISA in the presence of ecotin (10 nM), mono ecotin (400 nM), H-D-Phe-Pro-Arg-chloromethyl ketone (CMK; Bachem, Torrance, CA) (100 nM), or benzamidine (10 mM) was performed by standard ELISA methods, except that ecotin, mono ecotin, CMK, or benzamidine were added to each well and incubated 1 h before scFv was added.

**Immunohistochemistry (IHC).** IHC studies were performed on paraffin-embedded or frozen samples from prostate tissues. Sections were cut at 5  $\mu\text{m}$  and placed on pretreated slides (Fisher Scientific, Yorba Linda, CA). In the case of the paraffin-embedded sections, the antigen was retrieved and fixed using Citra (Biogenx, San Ramon, CA) according to the manufacturer's protocol. The frozen sections were fixed using a standard acetone fix method. Prior to immunostaining, sections were treated by peroxide block, avidin-biotin block, and protein block sequentially. Sections were incubated with each scFv and link at various concentrations. Peroxide-conjugated streptavidin label was applied. The antigen-antibody complex was localized by the chromogen 3-amino-9-ethylcarbazole. Sections were counterstained with aqueous hematoxylin before gelatin-mounting medium was applied.

## RESULTS

**Phage Selection.** Round 1 panning began with screening the scFv phage library (21) against immobilized, active hMT-SP1-P. Selection with two separate pools of washing solutions, namely, "standard" and "ecotin" pools, were carried out. The wash solution for the standard pool was TBS containing Tween 20. In the ecotin pool, ecotin, a macromolecular inhibitor specific for trypsin-fold serine proteases (28–31), was added to TBS containing Tween 20 as a competitive component to isolate more potent protease inhibitors. After washing, the surviving phage-displayed scFvs were incubated with *E. coli* cells and inoculated with helper phage, to generate a new repertoire for round 2. The numbers of surviving titers were  $10^5$  in the ecotin pool and

$1.5 \times 10^5$  in the standard pool out of the initial  $3 \times 10^{10}$  phage-displayed scFvs in each pool.

In round 2 panning, more elongated washing times were applied for both standard and ecotin pools. The numbers of titers of both pools drastically decreased; out of  $1.5 \times 10^{10}$  provided, only 90 survived in the ecotin pool and 165 in the standard pool. The surviving phage were incubated with *E. coli* cells, and those cells were grown and harvested. Plasmid DNA were isolated, digested with restriction endonucleases, and subcloned into a vector for scFv expression that includes a hexahistidine tag at the C-terminus. The expression vector harboring selected scFv genes were electroporated into *E. coli* cells that were subsequently plated onto LB agarose plates. For each pool, 96 colonies were picked, grown, and lysed.

ELISA was performed using the cell lysates containing scFv to estimate binding affinity of scFv to the target antigen hMT-SP1-P. From the standard pool, 24 scFvs were ELISA-positive, while only 7 scFvs were ELISA-positive in the ecotin pool. The variants that emitted the strongest ELISA signals, four scFvs from the standard pool and two from the ecotin pool, were chosen for further characterization. Because the ELISA signal is undetectable for scFvs expressed at extremely low levels, our system was capable at selecting the scFv variants possessing high binding affinity to hMT-SP1-P as well as reasonable protein expression levels.

**Isolation of the ScFvs and Sequence Analysis.** The variants that were characterized further were E1 and E2 from the ecotin pool and S1, S2, S3, and S4 from the standard pool. The letter "E" denotes the variant from the ecotin pool and "S" the variant from the standard pool. The sequences of the V<sub>L</sub> and V<sub>H</sub> regions that were determined by DNA sequencing are shown in Table 1. The six clones yielded six independent, unique sequences.

The library was originally constructed using 49 templates by shuffling 7 heavy-chain templates and 7 light-chain templates (21). As shown in Table 1, primarily VH3 templates were chosen for the heavy chain while a variety of templates were chosen for the light chain. The complementarity determining region (CDR) 3 of the heavy and light chains were designed to vary in the composition and size of the sequence. The number of amino acids in heavy chain CDR3 ranges from 15 to 19 residues and the light chain CDR3 ranges from 7 to 9. The CDR3 of the V<sub>H</sub> was randomized with 5–28 residues, and the CDR3 of the V<sub>L</sub> was randomized with 4–21 residues (21).

**Inhibition Analysis.** The purified scFvs were analyzed by SDS-PAGE and Coomassie Brilliant blue staining (Figure 1a), and size-exclusion chromatography (Figure 1c) to validate that the scFvs were pure monomeric proteins. To

Table 2: Inhibition Analysis

ScFv	$K_i^*$ (nM) <sup>a</sup> (hMT-SP1) <sup>b</sup>	$K_i^*$ (nM) (mMT-SP1) <sup>c</sup>	$K_i^*$ (mMT-SP1) $K_i^*$ (hMT-SP1)
E1	2 ± 0.14	>200	>100
E2	0.05 ± 0.01	40 ± 10	800
S1	129 ± 3	NI <sup>d</sup>	—
S2	> 500	NI	—
S3	40 ± 4	NI	—
S4	0.59 ± 0.02	>900	1525

<sup>a</sup> Inhibition analysis was performed at 25 °C. The protease concentration was 1 nM for all scFvs except E2. For E2 40 pM hMT-SP1 and 1 nM mMT-SP1 were used. The initial reaction velocities of human MT-SP1, mouse MT-SP1, and rat trypsin were measured in 50 mM NaCl, 50 mM Tris-HCl (pH 8.8), 0.01% Tween 20, 100 μM Spectrozyme tPA (methyl-sulfonyl-D-cyclohexyltyrosyl-glycyl-L-arginine-p-nitroanilide), and various concentrations of the scFvs. The  $K_m$  of Spectrozyme tPA for hMT-SP1 is  $31.4 \pm 4.2 \mu\text{M}$  (12).  $K_i^*$ s were evaluated by fitting to the equation for tight-binding inhibitors (27).  $K_i^* = K_i / (1 + [S]/K_m)$ , [S] = substrate concentration. All of these scFvs did not inhibit rat trypsin to any appreciable extent. <sup>b</sup> Recombinant protease domain of human MT-SP1 (hMT-SP1-P) was tested. <sup>c</sup> Recombinant protease domain of mouse MT-SP1 (epithin-P) was tested. <sup>d</sup> NI stands for "no inhibition".

determine the potency and specificity of the scFv to MT-SP1, inhibition assays were performed with the human MT-SP1 protease domain (hMT-SP1-P), mouse MT-SP1 protease domain (epithin-P), and rat trypsin. The initial reaction velocities were measured for several concentrations of the scFvs using Spectrozyme tPA as a substrate. Apparent inhibition constants were evaluated by fitting to a model of tight-binding inhibitors (27). The results are indicated in Table 2. The sequences of hMT-SP1-P and epithin-P exhibit 86.6% identity of amino acids. Since epithin-P shares such a high sequence identity with hMT-SP1-P, the difference in inhibition between these enzymes is a reasonable indicator of the selectivity of these scFvs. Rat trypsin has 35% amino acid sequence identity with MT-SP1 and was chosen as a control for general serine protease inhibition by the scFvs. We used the ratio of the apparent inhibition dissociation constant ( $K_i^*$ ) of hMT-SP1-P and epithin-P as a selectivity parameter.

All of the six scFvs inhibited hMT-SP1-P at far lower concentrations than epithin-P and did not inhibit rat trypsin to any appreciable extent. The selectivity for hMT-SP1-P over epithin-P was more than 100-fold with E1, 800-fold with E2 and 1500-fold with S4. Among these scFvs, E2 was the most potent inhibitor with a  $K_i^*$  of 50 pM for hMT-SP1-P and 40 nM for epithin-P. S4 was the most selective, inhibiting hMT-SP1-P with a  $K_i^*$  of 0.59 nM and epithin-P at greater than 900 nM. S1 and S3 moderately inhibit hMT-SP1-P but do not inhibit epithin-P measurably.

**Binding Kinetics.** The binding kinetics of the six scFvs to hMT-SP1-P was further characterized by surface plasmon resonance (Figure 2). The association ( $k_{on}$ ) and dissociation ( $k_{off}$ ) rates were measured, and the dissociation equilibrium constant ( $K_d$ ) was calculated (Table 3). For E1, E2, and S4, the  $K_d$  correlates well with the  $K_i^*$  values, while the  $K_i^*$  values of S1 and S3 were 27–100-fold higher than their  $K_d$  values. S4 exhibited an unusually fast association rate ( $1.2 \times 10^7 \text{ s}^{-1} \text{ M}^{-1}$ ), while E2 and E1 dissociate slower than other scFvs.

**Mode of Binding.** With the isolated scFvs, competition ELISA was performed in the presence of ecotin variants or

Table 3: Kinetics of Binding between Human MT-SP1 and ScFvs

ScFv	$k_{on}$ ( $10^6 \text{ s}^{-1} \text{ M}^{-1}$ ) <sup>a</sup>	$k_{off}$ ( $10^{-3} \text{ s}^{-1}$ ) <sup>a</sup>	$K_d$ (nM) <sup>b</sup>
E1	$0.63 \pm 0.02$	$1.1 \pm 0.2$	$1.8 \pm 0.3$
E2	$2.1 \pm 0.5$	$0.38 \pm 0.07$	$0.16 \pm 0.01$
S1	$1.5 \pm 0.3$	$1.9 \pm 0.1$	$1.3 \pm 0.4$
S2	—	—	>500 <sup>c</sup>
S3	$1.7 \pm 0.2$	$2.5 \pm 0.5$	$1.5 \pm 0.2$
S4	$11.5 \pm 2$	$5.8 \pm 0.4$	$0.51 \pm 0.06$

<sup>a</sup> From global fitting of the sensorgram data to a 1:1 Langmuir binding model in BIAevaluation 3.1 software. <sup>b</sup> Average and standard deviation of  $k_{off}/k_{on}$  for each repeat. <sup>c</sup> Estimated by steady-state affinity.

Table 4: Competition ELISA<sup>a</sup>

scFv	no ecotin	ecotin	mono ecotin	CMK	benzamidine
E1	+	—	—	—	+
E2	+	—	—	—	—
S1	+	—	—	—	—
S2	+	+	+	+	+
S3	+	—	—	—	—
S4	+	—	—	—	—

<sup>a</sup> ELISA was performed with hMT-SP1-P only and in the presence of ecotin (10 nM), mono ecotin (400 nM), H-D-Phe-Pro-Arg-chloromethyl ketone (CMK) (100 nM), or benzamidine (10 mM) with various concentrations of scFvs. Concentrations of the competitors were chosen at least 10 times higher than their  $K_i$  values against hMT-SP1.

smaller protease inhibitors, including a peptidyl chloromethyl ketone (CMK), and benzamidine (Table 4, Figure 3). Mono ecotin (30) is an engineered ecotin variant that was designed to be monomeric and binds only to the active site of trypsin-fold serine proteases inhibiting the enzyme activity of MT-SP1 at 75 nM  $K_i^*$ . In the presence of ecotin, mono ecotin, or CMK, only S2 was ELISA-positive, indicating that S2 binds a site where these inhibitors do not bind and the rest of the scFvs bind at or close to the active site. With benzamidine, which blocks the S1 pocket of MT-SP1, E1 and S2 were ELISA-positive, suggesting that these scFvs do not involve interactions with the S1 pocket.

**Immunoblotting.** Immunoblot analysis of the 6 scFvs were performed with hMT-SP1-P, and a prostate cancer cell line (PC-3) (26) that expresses the full-length MT-SP1. HeLaS3 cells were used as a control cell line that does not express MT-SP1. Among the scFvs, only S2 recognized the denatured antigen, as represented in Figure 1b.

**Immunohistochemistry (IHC).** The IHC examinations of a fraction of whole tumor tissues from randomly chosen cancer patients revealed that only S1 of the six scFvs tested stained MT-SP1 expressed in the tissues (Figure 4). It was indicated that MT-SP1 is localized in the luminal membrane in both the normal prostate tissue and tumor prostate tissue (Figure 4). Noteworthy is the shedding of MT-SP1 into the lumen (Figure 4b) in a metastatic prostate cancer tissue sample, suggesting MT-SP1 is shed from the surface of the epithelial cell.

## DISCUSSION

Technical advances in protein engineering have extended the original roles of antibodies in the molecular defense system by creating novel functions on the antibody scaffold (32–36). Since antibodies feature high-affinity binding with

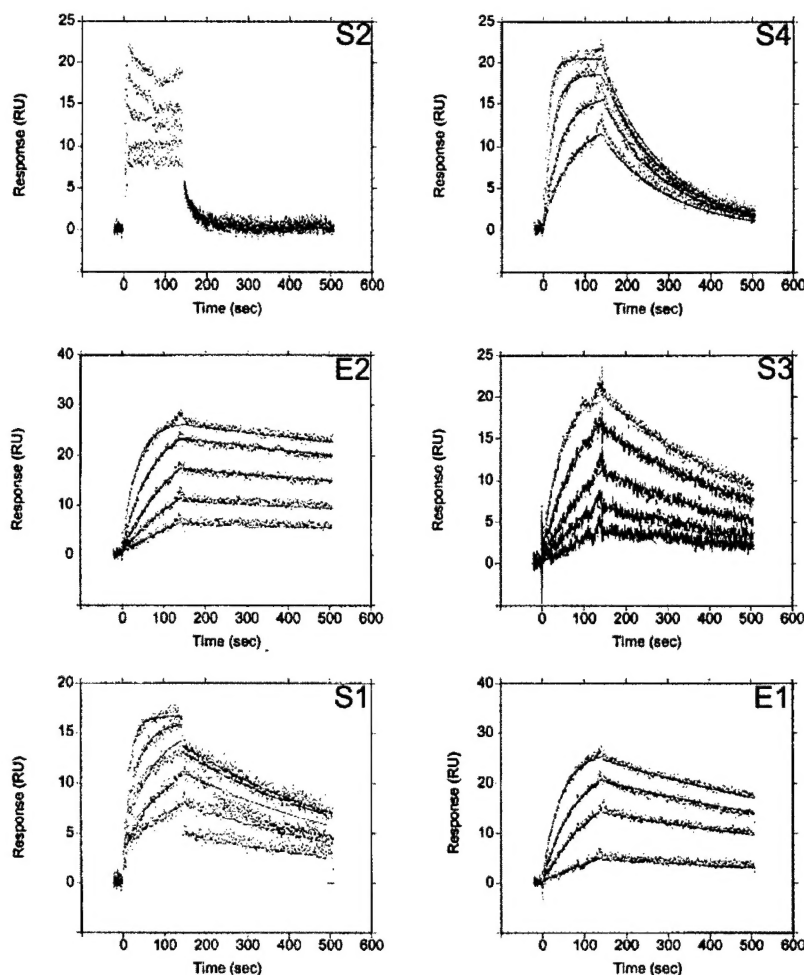


FIGURE 2: Binding kinetics of hMT-SP1-P and each scFv. The name of each scFv is denoted in the top right corner of each graph. The hMT-SP1-P (50 RU) was immobilized onto a CM5 chip. Each scFv was injected onto the CM5 chip at 50  $\mu\text{L min}^{-1}$ . Sensorgrams of surface plasmon resonance were obtained with concentrations of each scFv ranging from 0.2  $K_d$  to 5  $K_d$  of each scFv. The use of very low MT-SP1 density on the chip (50 RU) minimized the mass transfer and rebinding problems affecting the  $k_{on}$  value. Single injections of S4 (2 nM) at 5, 15, 50, 75, and 100  $\mu\text{L min}^{-1}$  showed mass transfer-associated deviations at 5  $\mu\text{L min}^{-1}$ , but the sensorgrams for 15, 50, 75, and 100 were essentially identical. Determination of kinetic parameters for S4 was performed at 50 and 100  $\mu\text{L min}^{-1}$  flow rates on the same chip. We obtained similar values with a small deviation that reflects a slight difference in protein concentrations between the experiments.

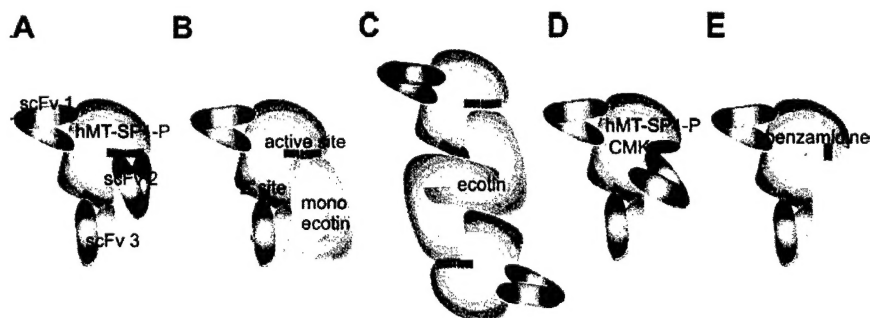


FIGURE 3: Mapping scFvs using ecotin variants. (A) ScFvs 1 (S2) binds to neither the active site nor the secondary site of hMT-SP1-P. ScFv 2 (E1, E2, S1, S3, and/or S4) binds to the active site and scFv 3 binds to the secondary site. The scFvs that bind to the enzyme can be ELISA-positive. (B) The primary site is blocked by mono ecotin, excluding scFv 2. (C) Ecotin competes off both the blue and scFvs 2 and 3. (D) CMK occupies a small area covering the active site. (E) Benzamidine only interacts with the S1 pocket in the active site.

outstanding selectivity, they have great potential to be efficient inhibitors against virtually any biocatalyst. Although inhibitory antibodies targeting proteases have been reported to date, antibodies exhibiting potency and selectivity have not been described (37–40). Developing selective and potent inhibitors for proteases is difficult because a target protease

is frequently coexpressed with other similar proteases that differ only slightly in sequence and specificity. One example is the membrane-type serine protease family that currently includes seven human members and the number is still increasing (4). The antibody scaffold is an attractive choice for the development of highly potent and selective inhibitors

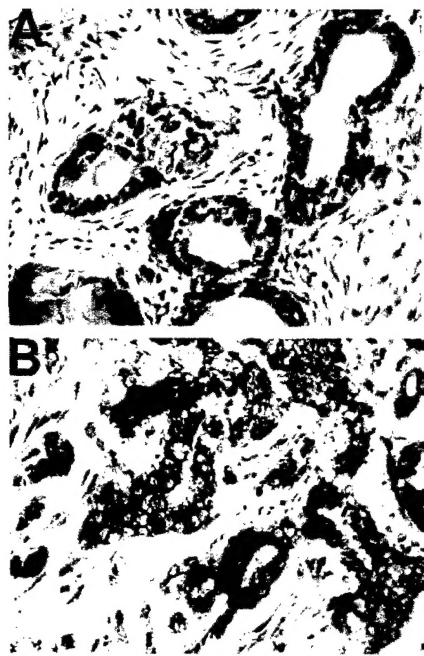


FIGURE 4: Immunostaining (red) of tissues with the S1 detected MT-SP1 in the luminal membrane from a normal prostate (A) and in the luminal membrane as well as inside of the lumen in a prostate cancer (B).

for this family since it can immunologically distinguish between closely related proteins. Furthermore, antibodies can easily provide diagnostic and therapeutic reagents. If successful, the method itself can be applied to other enzyme families such as matrix metalloprotease families and protein kinase families.

Regulation of cell surface enzyme activity provides promising specific targets for antibody-based inhibitors. We applied an antibody phage display method to obtain potent and selective antibody protease inhibitors against MT-SP1, a cell surface protease that has emerged as a potential key factor in triggering a proteolytic cascade involved in cancer metastasis. A naïve, synthetic, human antibody library (21) was used as a source of single-chain antibodies to screen against hMT-SP1-P.

Since nonspecific binding and low initial concentrations of desired species could easily result in isolating undesired scFv species, an affinity washing procedure was developed. Ecotin was added to the washing solutions to raise stringency in preventing reassociation of released phage by binding to open sites on hMT-SP1-P and the plate. The ecotin used in this affinity washing procedure was an engineered dimeric variant that has a  $K_i^*$  of 50 pM for MT-SP1. Although it is a slight difference, ecotin-washing reduced the number of scFvs surviving after each round of panning as well as the number of ELISA-positive variants. Interestingly, the E1 and E2 that were derived from the ecotin-washing pool dissociated slower than other scFvs and E2 was the most potent variant in inhibition as well as in binding (Tables 2 and 3). This implies that the selection pressure of the affinity washing conditions may be the dissociation rate ( $k_{off}$ ) allowing for selection of scFvs with slow off-rates. Adding a competitive inhibitor in the wash solutions may not interfere with the tightly bound scFvs that dissociate slowly. The use of ecotin in one step of the panning procedure to

result in a more potent inhibitor suggests other possibilities. For example, a similar application in the phage binding or elution steps could result in inhibitors with different modes of binding with increased potency and selectivity.

Inhibition analysis (Table 2) showed that both potency and selectivity was very high in the selected scFvs. The level of potency was up to 50 pM (E2), and the level of selectivity was maximized up to 1500-fold (S4). In the scFv library, the randomized regions are CDR3s of  $V_H$  and  $V_L$  due to higher solvent exposure. Thus, the variations on these two loop regions, in particular of  $V_H$ , enabled these scFvs to clearly discriminate the slight sequence difference between the human and mouse MT-SP1s.

A competition ELISA was devised employing ecotin and mono ecotin to map epitopes on MT-SP1 (Figure 3, Table 4). To narrow down the possible binding modes of these scFvs, the competition ELISA was also performed in the presence of a peptidyl chloromethyl ketone derivative or benzamidate. The ecotin inhibits hMT-SP1-P at 50 pM  $K_i^*$  with two binding sites: the active site and a secondary site remote from the active site (29) while mono ecotin binds only to the active site with a  $K_i^*$  value of 75 nM. The scFvs that are positive in both the ELISAs with the ecotin and the mono ecotin do not bind to either the active site or the secondary site of the protease unless these scFv bind more tightly than the ecotins. These scFvs could be useful allosteric inhibitors of MT-SP1. The scFvs that are positive in the ELISA with mono ecotin and not with ecotin may bind to the protease secondary site or compete more effectively with the mono ecotin for the binding site. This competition ELISA method allowed rapid determination of the binding mode of the scFvs and prioritized our future applications of their binding and inhibitory properties.

A combination of the inhibition assays, binding kinetic analysis, and the competition ELISA provides a more comprehensive description of the binding modes of the scFvs. E1 may not interact with the P1 site because it was positive in the competition ELISA in the presence of benzamidate. S1 competes with monomeric ecotin, but its  $K_i^*$  value is 100 fold higher than its  $K_d$  value. This suggests that the epitope recognized by S1 may be a different site which is near the active site and not the same site recognized by E2 or S4. Alternatively, since S1 is a strong binder ( $K_d = 1.3$  nM) and immunostains fixed tissue slides while other scFvs did not, S1 could recognize a linear epitope still present in MT-SP1 denatured by the fixing process. In contrast, other scFvs might require three-dimensional epitopes. The S3 scFv has a 27-fold higher  $K_i^*$  than the  $K_d$ . This, in conjunction with the ELISA results, suggests that S3 is likely to act like S1. However, S3 is not effective in immunostaining. Thus, the binding site of the S3 is not the same as the rest of the scFvs. Since the mono ecotin covers a broad area spanning 1900 Å<sup>2</sup>, it is also possible that S3 may bind to a site near to but not exactly the active site (41).

One reason so many tight-binding antibodies appeared to target the active site may be because it is located in a protein cleft, which has intrinsic physicochemical properties that are favorable to protein-protein interactions (42). In this cleft, there are several  $\Omega$ -loops that are positioned on the surface of the protein so as to be exposed and serve as epitopes for the possible binding in several different modes (43, 44). Recently, the catalytic domain of human MT-SP1 was



crystallized in complexes with benzamidine and basic pancreatic trypsin inhibitor (BPTI), and the structures were solved to 1.3 and 2.9 Å, respectively (45). These structures illustrate that hMT-SP1-P has a unique 60s-loop in the active site that is nine amino acids longer than in other trypsin-fold serine proteases, which may strongly influence the interaction between the enzyme and scFvs.

As indicated in Tables 2 and 3, S2 inhibits and binds to the antigen with far lower affinity than the other scFvs. ELISA experiments (Figure 3, Table 4) showed that it binds to neither the active site nor the secondary site of hMT-SP1-P. Because S2 did not have to compete for binding to the antigen with the ecotins, this scFv may have survived the affinity-based washing conditions. However, the weakness of the binding affinity of this scFv suggests another possible selection mode. This scFv may have been selected against a denatured epitope to which S2 bound with high affinity during panning or immunoblotting, yet lost its affinity on interacting with native hMT-SP1-P in enzyme activity assays or binding kinetic analyses. Although less likely, several other reasons are possible. S2 could be highly represented in the initial library or expressed at high levels on phage. It may be displayed polyvalently on phage thus allowing an avidity contribution to selection. Alternatively, the amine coupling used for the Biacore experiments may inactivate the S2 binding site giving rise to an apparent weak affinity.

Examination of human tissue sections by IHC showed that the scFv S1 stained human prostate tissues. MT-SP1 is present in normal prostate tissue (Figure 4a) as well as prostate cancer tissue (Figure 4b). However, in addition to being present at the membrane surface of the lumen, it also appeared in the luminal space of the prostate tumor section suggesting a shedding event. The form of MT-SP1 that is released from the membrane surface may prove to be a useful diagnostic marker for prostate cancer.

In comparison to efforts to develop small molecule inhibitors, in vitro selection of scFvs provides a rapid, general, and robust means to produce potent and selective inhibitors for proteases that are very similar to one another. Furthermore, these scFvs can be used as diagnostic or prognostic reagents. Finally, the therapeutic role of selective protease inhibition can be evaluated in appropriate cancer models by studying the effects of MT-SP1 inhibition on cancer development in vivo. Should the scFvs be sub-optimal to develop as therapeutic agents, an atomic-level understanding of the binding modes of these molecules with their cognate protease may reveal principles that will guide the rational design of small-molecule inhibitors.

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